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Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

M. Sc.

Year this degree conferred — Année d'obtention de ce grade

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INDUCTION OF RESISTANCE AGAINST SCHISTOSOMA MANSONI

By

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

EDMONTON, ALBERTA

FALL, 1984

THE UNIVERSITY OF ALBERTA

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ABSTRACT

The induction of resistance against Schistosoma mansoni was investigated using parasite derived antigens, immunologic adjuvants and monoclonal antibodies. The resistance to reinfection by Schistosoma mansoni described in mice with chronic Schistosomiasis has been combined additively with the non-specific acquired resistance induced by mycobacterial adjuvants. This study describes the induction of resistance to S. mansoni with parasite preparations and the augmentation of this resistance with bacilli Calmette-Guerin (BCG). CFI mice were immunized subcutaneously with 2000 freeze thawed schistosomula (FTS) either alone or in combination with BCG (10^7 CFU). Resistance to S. mansoni was assessed by the reduction in schistosomula recovered from the lungs of immunized mice after percutaneous challenge with 500 cercariae. Lung recovery of schistosomula was reduced 26% in mice immunized with FTS ($P < .001$). BCG alone induced 44% protection ($P < .01$) but when combined with FTS lung recovery was reduced by 62% ($P < .01$). Antibody levels were detectable by enzyme linked immunosorbent assay (ELISA) in immunized mice but not in controls (absorbance of .323 vs .024). Peritoneal macrophages from FTS immunized mice did not mediate killing of schistosomula while macrophages from BCG treated mice killed 32% ($P < .05$). The resistance induced by FTS was replicated with protein extracted from schistosomula by 1% Triton, 0.5 mM

phenyl methyl sulfonyl fluoride and 0.2 mM iodoacetamide (TSE). Immunization of mice with 100 μg of TSE induced 25% protection ($P < .02$) as determined by lung recovery of schistosomula 5 days after challenge with cercariae. A protective antigen was also defined using a monoclonal antibody. Anti Schistosoma mansoni monoclonal antibodies were raised by standard fusion techniques from Balb/C mice immunized with freshly transformed schistosomula and Freund's adjuvants. Positive clones were identified by ELISA adapted for adult worm antigen preparation. One of these positive clones 31-3 B6 was also positive by ELISA adapted for egg antigen and was shown to be active against schistosomula as demonstrated by indirect immunofluorescence. The in vitro and in vivo activity of this IgM antibody was investigated. Antibody 31-3/B6 mediated in vitro killing of schistosomula by peritoneal exudate cells ($29 \mp 3\%$, $P < .025$) which was augmented by complement ($P < .025$). By passive transfer this IgM induced 30% protection in treated mice as assessed by the reduction in lung recovery of schistosomula 5 days after challenge with S. mansoni ($P < .01$). The adult worm antigen recognized by western blot had an apparent molecular weight of 35,000.

The investigation demonstrates that the additive nature of resistance previously demonstrated in chronically infected mice treated with adjuvants can be duplicated with a dead

parasite preparation and an adjuvant. Also described is an IgM monoclonal antibody which recognizes schistosomula, adult worm and egg antigens. This antibody induces protection *in vivo* and schistosomula killing *in vitro*.

ACKNOWLEDGEMENTS

I wish to thank Dr. H.T.G. Williams, Chairman of the Department of Surgery, University of Alberta and Dr. A. A. F. Mahmoud, Head of the Division of Geographic Medicine, Case Western Reserve University for the opportunity to conduct research work in Cleveland.

I am grateful to Dr. Mahmoud who oversaw my work at Case Western Reserve University and Dr. J. Russel who was my supervisor at the University of Alberta.

I wish to acknowlege Dr. G. R. Olds who performed the hybridoma fusion for me and Dr. C. King who collaborated with me on the *in vitro* killing assays. The technical assistance and life cycle maintenance by Mrs. E. Moss and Mr. P. Peters was essential for the performances of this study. I also wish to thank Karen Clarke for typing and processing the thesis.

Finally I wish to acknowledge with thanks the Fellowship awarded by the Alberta Heritage Foundation for Medical Research and the partial support provided by the Edna McCornell Clark Foundation.

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CHAPTER 1 INTRODUCTION

The worldwide distribution and extensive pathologic changes produced by schistosomiasis makes it the single most important helminthic infection of humans (1). It is estimated that 200 million people in Africa, South America, Asia, and the Caribbean are infected with one of the five related species of the genus Schistosoma. Schistosoma mansoni, S. hematobium and S. japonicum are the most widespread and important species (2) causing human disease. S. mansoni is endemic in Africa and the Caribbean and Middle East. S. hematobium is seen in Africa and the Middle East while S. japonicum is endemic in Asia.

LIFE CYCLE

The parasite traverses six dramatic developmental changes during its life cycle. The cercaria is an aerobic free swimming larva which penetrates the skin of the definitive mammalian host through a combination of proteolytic enzymes and muscular activity (3). Upon skin penetration the cercaria loses its tail, becomes an anaerobe and its limiting membrane transforms from a conventional trilaminate to a heptolaminate structure (4). The resulting schistosomula migrate from the subcutaneous tissue through the lung and then to the liver (5)

where they mature into the dioecious adults. From the liver the organisms migrate to their final habitat which is the inferior mesenteric veins for S. mansoni, the species used in this study. Approximately 300 eggs are expelled daily by the female. Many of these are retained in host tissues and another portion passes to the outside. When the egg comes to lie in close contact with a mucosal surface it penetrates into the lumen of the gut or bladder and is subsequently expelled from the host. Eggs that contact fresh water hatch and release the ciliated miracidia which search out and penetrate the specific snail appropriate to the species. The snail is the intermediate host where the resulting sporocyst undergoes asexual reproduction and over a two month period thousands of cercariae are released (1). The cycle is complete when these cercariae penetrate the skin of the definitive host.

PATHOGENESIS

Much of the disease due to Schistosoma mansoni infection, results from the immunologic response of the host to the parasite life cycle stages: the penetration of cercariae through the skin, the migration of the schistosomula, the onset of oviposition and the retention of eggs (1). Many cercariae die upon skin penetration (6); the cercarial antigens elicit both an immediate and a delayed type of

hypersensitivity reaction. The cutaneous manifestations are characterized by relatively mild edematous reaction on primary exposure and by a maculo papular itchy rash on subsequent encounters (7, 8). The initiation of oviposition by the mature female results in the release of antigen and leads to the formation of immune complexes. Previously unexposed individuals develop a serum-sickness-like illness, Katayama fever (9) which is characterized by high spiking fevers, cough, arthralgia, urticaria, tender hepatosplenomegaly, lymphadenopathy and peripheral eosinophilia (1, 7).

The response of the host to retained eggs and their secreted antigens includes eosinophil and mononuclear cell infiltrates, edema, granuloma formation, fibrosis and vascular obstruction (1). Disease from established S. mansoni infection is seen in sites where eggs are deposited. A considerable proportion of S. mansoni eggs are retained in intestinal tissue and an estimated 33% reach the liver. The colonic mucosa may show minute hemorrhages, ulcerations or diffuse polyposis. The liver usually shows major pathologic change. A major effect of this liver disease is presinusoidal block in portal blood flow, and the development of portosystemic collaterals due to portal hypertension. Marked splenomegaly results from the congestive element contributed by portal hypertension and the proliferative cellular response to the parasite (7).

SCHISTOSOMIASIS CONTROL

Control of the schistosomiasis theoretically requires that the schistosome life cycle be broken. Control of the environmental stage of the parasite by using molluscacides to kill the intermediate host has not been strikingly successful (10). Repeated doses of molluscacide must be administered to involved bodies of water, indefinitely, to control these snails (11, 12). Irrigation projects provide ideal breeding places for the snail resulting in the spread of this disease associated with agricultural developments (5, 10).

The life cycle could in theory be broken by treatment of infected humans and proper treatment of human excreta (8). Mass treatment programs are designed to control infection and disease manifestation, which are most prevalent in the heavily infected portion of the population. These programs are becoming more practical with the development of safer drugs and are presently being evaluated. Limiting contact with contaminated water is very difficult as populations in endemic areas often have only one source of water for washing, drinking and irrigation (10). At present there are no measures either pharmacologic or immunologic which will prevent this parasite from infecting human beings in contact with contaminated water or that will increase immunity or resistance to infection.

RESISTANCE TO INFECTION

Schistosoma present a challenge to host defenses because of their size (13), their multiple developmental stages (14), and their ability to modulate their surface antigens (4). The host response to Schistosoma infections is complex involving humoral, cellular and phagocytic components (13). The outcome of infection in permissive hosts such as humans and laboratory mice varies from death of the parasite in the skin, or enroute to their final habitat (7) to maturation of the parasite and initiation of oviposition (8). The degree of infection in human beings is measured by the output of eggs in excrement (16), in laboratory animals by the number of schistosomula recovered from the lungs or the number of adult worms recovered from the portal tract are the measurements used (17). Evidence for the existence of different forms of resistance to S. mansoni such as innate, non-specific and specific will be presented.

a) Innate Resistance

Susceptibility to infection with Schistosoma is dependent on the parasite as well as the host. Man is susceptible to S. mansoni, S. hematobium and S. japonicum whereas avian schistosomes are unable to mature in a human host. Mice and hamsters are very susceptible to S. mansoni, whereas rats and

rabbits are not. This species specific defence against schistosomiasis is termed innate resistance (18).

i) In vivo investigations

Experiments were performed to evaluate the susceptibility to S. mansoni infection in different mammalian species (19). Thirteen mammals were classified as either very susceptible, partially susceptible, poorly susceptible or resistant. The very susceptible group which included the laboratory mouse, woodchuck and squirrel developed liver disease, allergic phenomena and allowed the parasite to complete its life cycle. The partially susceptible group included the oppossum which had a low yield of eggs in the stool, the rabbit which had severe liver disease but allowed no access of eggs to the colon and the skunk which deposited eggs in the small intestine where they appeared to degenerate. Stunted adult worms and poor egg production, were the findings in the poorly susceptible group which was comprised of the mole, chipmunk, racoon and nutria. The resistant group included the rat which allowed development of a few stunted adults, the muskrat which stopped development of the schistosome before adulthood and the fox which did not allow the parasite to enter the subcutaneous tissues.

ii) In vitro investigations

In vitro experiments were carried out to evaluate the role of the monocyte in species related innate resistance to

Schistosoma mansoni (18). Rat, guinea pig and rabbit macrophages, killed 21±2.3%, 15±9.6% and 17±5.5% of schistosomula. Mouse and hamster macrophages failed to increase schistosomula mortality above the 5±1.1% level demonstrated in cell free media.

iii) Mechanisms

The mediators of macrophage killing of schistosomula were investigated (18). The addition of L-arginine inhibited the killing by rabbit and rat macrophages suggesting a role for the enzyme arginase in this killing. The killing of schistosomula by the rat macrophage was significantly inhibited by catalase suggesting a role for oxygen intermediates in schistosomula killing by rat macrophages.

The in vitro studies presented clearly demonstrate the direct relationship between the in vitro ability of macrophages to kill schistosomula and the level of innate resistance to infection with S. mansoni demonstrated in different species.

Innate resistance plays a part in resistance to S. mansoni. The susceptibility of different mammals to parasitism depends on host mechanisms which may be effected at the skin, lung or portal veinous system.

4b. Specific Resistance

Man acquires specific resistance to many bacterial and viral diseases. Specific resistance is the function of the

immune system which discriminates foreign molecules from the hosts own molecules. Antigenic challenge triggers a co-ordinated effector response primarily involving macrophages and lymphocytes. Antigen is presented to T-lymphocytes after being processed and incorporated into the cell membrane of host macrophages. Only the subset of macrophages with immune associated (Ia) protein on their surface have the ability to interact with T-cells (20).

After interaction with the macrophage, T-cells can then act directly in response to the antigen, stimulate effector macrophages, or stimulate other classes or subclasses of lymphocytes. Stimulated lymphocytes produce clones of memory cells which respond to a second challenge by the same antigen.

In the case of schistosomiasis, this powerful immunity which destroys invading bacteria and viruses is unsuccessful. Some would argue therefore that acquired resistance to schistosomiasis does not develop (15).

i) Epidemiologic evidence for specific resistance

Epidemiologic evidence does suggest that humans do develop specific resistance to Schistosoma. Subjects in endemic areas are repeatedly exposed to infested waters yet there is not a steady increase in adult worm burden, resulting in severe morbidity and mortality. Most individuals in endemic areas do not develop overwhelming schistosomal

infection (21). Intensity of infection as measured by egg output reach a peak in the teenage years and declines with advancing years. In one study in a village in Kenya an egg output of > 400 eggs/g feces was defined as heavy S. mansoni infection. The mean age for this heavily infected group was 14 years (10). The egg output in adults in endemic areas is lower, suggesting a reduction in worm burden and the development of immunity. This immunity in the presence of infection is termed concomitant immunity. The nature of specific resistance to S. mansoni has been investigated using laboratory models.

ii) In vivo investigations

Sher et al (17) demonstrated decreased recovery of schistosomula from the lungs of mice which had been previously infected with small numbers of cercariae. Protection was shown in 12-15 week infected mice by challenging them with 500 cercariae and recovering lung schistosomula 5 days later. The recovery in six strains of mice showed a decrease ranging from 50.4-75%. This decrease in worm burden was confirmed by the recovery of adult worms from the portal system at six weeks; the decrease was 74.2% over uninfected controls. Thus mice with a previous infection demonstrate resistance albeit incomplete, to re-infection with S. mansoni. This immunity may be similar to the concomitant immunity seen in man.

If acquired resistance is an immune response to parasite antigens, transfer of this response should be possible (22). Serum from mice with an S. mansoni infection of 12-15 week was injected intravenously into uninfected mice, subsequently the animals were challenged with cercariae. The lung recovery of schistosomula in the chronically infected animals showed 65% protection, the protection in mice given immune serum was 41%. Successful transfer of immunity depends on the immune status of the host and the quantity of serum given. Serum from three to six week infected donors or in quantities less than 0.5 ml failed to induce protection. Attempts to transfer immunity on the third day after percutaneous challenge with cercariae and attempts at transfer of immunity with spleen or lymph node cells were unsuccessful (summary in Table 1).

These results demonstrate that challenge infections are susceptible to the effects of passively administered serum from 12-15 week mice if the serum is administered at the time the parasite penetrates the host.

Further in vivo investigations demonstrated a role for the eosiniphil in acquired resistance to S. mansoni . Mahmoud et al (23) raised rabbit antisera specific for murine eosinophils, lymphocytes, neutrophils and monocytes. Groups of chronically infected mice were given one of the four antisera prior to and following percutaneous exposure to 500

cercariae. As is demonstrated in Figure I, the mice which had anti-eosinophil serum lost the partial immunity expected with chronic infection. Further experiments demonstrated that passive transfer of immunity with serum was abrogated by prior depletion of the animal's eosinophils. These experiments clearly demonstrate the importance of both humoral factors and the eosiniphil in acquired resistance.

iii) In vitro investigations

In vitro investigations have allowed the investigation of the role of different leukocytes and humoral factors in the immune response to schistosomiasis. Schistosomula are obtained by in vitro cercariae penetraion of isolated mouse or rat skin (6). The best assays for schistosomula killing use morphologic and biologic criteria. Viable schistosomula are motile, exclude toludine blue or methelene blue dyes, and mature into adults upon injection into susceptible hosts. Anwar et al (24) examined the capacity of human eosinophils, neutrophils, and mononuclear leukocytes to kill schistosomula in vitro. They found that antibody alone, complement alone or antibody plus complement in combination with granulocytes or monocytes killed schistosomula. Antibody combined with complement was superior to complement alone, which was better than antibody alone in mediating cytotoxicity. The eosinophil was superior to the neutrophil in mediating killing when

complement was present but equally effective to the neutrophil when antibody was the only humoral factor present (see Table 2). The eosinophil has been demonstrated to be effective against S. mansoni both in vivo and in vitro but it must be noted that the neutrophil and monocyte were also effective in vitro mediators of schistosomula killing.

Studies with rat monocytes demonstrated that they could kill schistosomula in vitro if the immune serum contained IgE. This suggests a role for IgE in protective immunity in addition to its established role in immediate hypersensitivity (25).

The basophil has also been shown to be involved in host response to schistosomiasis. Treatment of chronically infected mice with compound 48/80 depletes basophils and results in the reduction of the resistance to re-infection by 95% (26). Basophils in the presence of complement adhere to schistosomula (27). The basophil products, eosinophil chemotactic factor and histamine preferentially attract human eosinophils in vitro. Enhanced complement mediated killing of schistosomula by eosinophils results from the presence of the basophil derived mediators (28). IgE release of basophil chemical mediators which is seen in helminthic diseases may amplify the parasiticidal properties of eosinophils (29).

The mechanisms of protective immunity in schistosomiasis are complex. The eosinophil has been demonstrated to be important *in vivo* and *in vitro*, however roles for the neutrophil, monocyte and basophil have been established. Complement and antibody are essential for the mediation of this cellular cytotoxicity.

iv) Mechanisms

The molecular mechanism of antibody dependent, cell-mediated killing of schistosomula involves the adherence of the antigen binding portion (Fab) of the antibody molecule to the parasite and the constant portion (Fc) of the antibody molecule to the granulocyte. After binding, the granulocytes release enzymes which break the tegument of the parasite and in the case of the eosinophil irreversibly bind the parasite (30, 31). Macrophages which have been sensitized by IgE immune complexes adhere to the target and release lysosomal enzymes.

The rate of H_2O_2 production by neutrophils, eosinophils and basophils was measured upon incubation with schistosomula, or in the presence of complement or specific antibody (32). Both the neutrophil and eosinophil release of H_2O_2 is increased in the presence of complement and further augmented by immune serum (see Figure 2). The production of H_2O_2 correlated with *in vitro* killing of

schistosomula. The presence of both opsonins led to 50% parasite killing and a 500% increase in H_2O_2 production. Catalase inhibited H_2O_2 production and leukocyte mediated killing.

Granulocytes from individuals with chronic granulomatous disease produce little or no oxygen intermediates. Neutrophils from these individuals lacked the ability to effect parasite death; their eosinophils however did kill a significant but reduced number of schistosomula. These experiments indicate that oxygen intermediates mediate killing by granulocytes and that eosinophils have oxygen independent mechanisms also.

c) Non-Specific Resistance

Resistance to intracellular organisms including bacteria (33), viruses (34) and protozoa (35) can be acquired non-specifically. This form of host defence has also been shown with Schistosoma mansoni (36) which is an extracellular parasite. Non-specific resistance can be defined as protection against an infectious agent which results from exposure to unrelated antigens (37).

i) In vivo investigations

Non-specific resistance to multicellular organisms was first demonstrated by Mahmoud, et al. (36). Infection of CFI mice with Toxoplasma gondii resulted in 35% protection against

infection with S. mansoni. In studies using intravenous BCG (Tice strain), 2 X 10^7 Colony forming units (C.F.U.) resistance against Schistosoma mansoni was again demonstrated even when BCG injection was delayed until three days after cercarial challenge. A summary of these results is displayed in Table 3. Effective resistance to S. mansoni did not result if the dose of BCG was reduced by a factor of ten to 2 X 10^6 C.F.U. At this dose the number of schistosomula recovered from the lung were 110% of controls (38). The non specific resistance obtained in mice with BCG can be completely abrogated by prior administration of antithymocyte globulin (39), indicating a role for the T-cell in non specific resistance.

The resistance conferred by BCG has also been reproduced by natural cord factor and trehalose dipalmitate, which are non-living extracts or synthetic products of the mycobacterium cell wall (40) (see Table 4).

ii) In vitro investigations

Peritoneal macrophages obtained from BCG or Corynebacterium parvum treated mice, but not from proteose peptone or thioglycolate injected animals, have the capacity to kill schistosomula in vitro (41). At 12 hours macrophages from BCG-treated mice killed 25∓9% of incubated schistosomula; the corresponding figure for C. parvum was 40∓3%.

iii) Mechanisms of non-specific resistance

The mechanism of non-specific resistance was first investigated by Mackaness (42) using bacterial infections for his model. Brucella infected mice were challenged with Listeria monocytogenes at different times during the course of Brucella infection. When delayed-type hypersensitivity is at its height 99.9% of Listeria present in cultures were inactivated. Table 5 shows that at day 18 the number of colony forming Listeria organisms was markedly decreased in the Brucella infected animals. Microscopic examination of macrophages demonstrated that those from Brucella infected mice contained more intracellular Listeria than normal macrophages. This study demonstrated that non-specific resistance to Listeria is conferred by Brucella infection and that the macrophage is the most likely effector of this mechanism (42).

Further evidence for the role of the macrophage comes from the in vitro studies of the interaction between macrophage monolayers and newly transformed schistosomula (41). The killing in vitro has been shown to be mediated by a soluble substance released in the culture containing activated macrophages. These supernatants killed 51±3% of the schistosomula; the controls showed no significant killing of schistosomula. The effector molecule was delineated by

comparing the arginase levels in supernatants of C57 BL/6J mice macrophage cultures which kill 30∓5% of schistosomula when stimulated by C. parvum and BABL/c mice macrophage cultures which do not kill schistosomula when treated in the same manner (43). As seen in Table 6, activation of the macrophage was associated with increased arginase production in C57 BL/6J mice but not in BALB/c mice. There was a linear relationship between arginase level and killing of schistosomula. Killing was not inhibited by superoxide dismutase or catalase suggesting that the mechanism in this case is oxygen independent.

iv) Genetic basis of non-specific resistance

In CBA mice, BCG confers resistance against Babesia and Plasmodia, but no resistance was conferred against schistosomiasis. The genetic basis for this difference has not been defined (37).

Various strains of mice showed significant difference in expressions of BCG-induced resistance to S. mansoni. C57 BL/10 mice (haplotype $H\text{-}2^b$) shows high non-specific resistance to S. mansoni infection. Congenic B10A and B10-D2 mice have the C57 BL/10 genetic background but with $H\text{-}2^a$ and $H\text{-}2^d$ haplotypes. Both of these strains were significantly protected. The BALB/c mouse (haplotype $H\text{-}2^d$) shows no non-specific induction of resistance to S. mansoni. The BALB/B10

mouse carries the H-2^b haplotype on the nonresponder background to the BALB/c mouse. This mouse is a non-responder like the BALB/c mouse, rather than a higher responder like the C57 BL/10 mouse even though it has the same H2 haplotype. Thus genes in the major histo-compatibility complex do not seem to be involved in non-specific protection against S. mansoni infection (44).

Induction of non-specific resistance depends on the characteristics of the host, the inducer and the agent challenging the host. This process requiring sensitized T-lymphocytes and nonsensitized macrophages (45). The messenger between T-lymphocytes and the macrophage is the lymphokine (46). This mediation results in the activation of macrophages which can then kill helminths of the genus Schistosoma as well as other organisms. Non-specific resistance has been stimulated by bacterial (33) viral (34) and protozoan (35) infections as well as synthetic bacterial products (40). Resistance to Schistosoma can be induced through both specific and non-specific mechanisms. The possibility of adding these two mechanisms were investigated. Mice with 14 week infection with Schistosoma mansoni were given trehalose dipalmitate as a stimulator of non-specific resistance. Thirty percent protection resulted from the adjuvant in uninfected animals however the recovery of adult

worms was reduced 78% in the chronically infected animals which had also received the adjuvant. This demonstrated the additive nature of two protective mechanisms which if exploited, could be of practical use in preventing schistosomiasis (40).

IMMUNIZATION

Numerous antigen preparations have been administered to laboratory animals in attempts to produce protective immunity agains schistosomiasis. These include live vaccines, attenuated by irradiation and non living preparations, usually homogenates of different developmental stages.

a) Irradiated vaccines

The most consistently successful preparations have been whole living cercariae or schistosomula which have been attenuated by irradiation (47). The majority of these studies have used cercariae but studies with irradiated schistosomula have also shown protection (48).

i) Low dose irradiation

Studies were performed to determine the dose of irradiation that would result in the most effective vaccine. In an early study the effect of varying doses of radiation on S. mansoni viability and fertility was performed. It was noted that cercariae that were exposed to 2,500 rads were

sexually sterilized but could mature into adult worms. Cercariae that received 5,000 or more rads did not survive to maturity. Mice were then vaccinated with cercariae treated with 2,500, 5,000 and 10,000 rads. Mice that were vaccinated with cercariae that had been treated with 2,500 rads showed 85% protection against challenge infections, while those vaccinated with cercariae treated with 5,000 rads showed 40% protection. No protection was induced with cercariae that received more than 5,000 rads. These experiments demonstrated a high degree of protection in the 2,500 rad irradiated cercariae vaccine which is the vaccine that allowed the worm to mature in the portal system of the host (49). Prolonged survival after receiving lethal doses of S. mansoni has been demonstrated in mice immunized with irradiated cercariae (50).

An irradiated cercariae vaccine has been tested, in a field study with cattle, in the Sudan (51). Fifteen percent of vaccinated calves died of schistosomiasis compared to a 50% mortality in unvaccinated calves. The mean worm burden in the control group was 1,418, in the vaccinated group 341. This protection of 75% suggests that Schistosoma bovis could be controlled by vaccination.

ii) High dose irradiation

Cercariae exposed to low doses of radiation migrate through the lungs and liver resulting in inflammatory changes

(53). Immunization with cercariae exposed to high doses of irradiation do not give these untoward effects (47) thus making a highly irradiated vaccine, safer and consequently more desirable. Immunization of Rhesus monkeys with highly irradiated cercariae (24,000 - 48,000 rads) did give good protection but required higher numbers of cercariae than in vaccines irradiated with low doses(52). In another murine study the highly irradiated cercariae gave better protection than cercariae that received lower doses. This data contradicts the studies in which greater protection resulted with the administration of cercariae that had been irradiated with 2,500 - 3,000 rads (54).

iii) Mechanisms

The mechanism of protection induced by immunization with irradiated larval vaccine have been investigated. With the use of skin and lung recovery of schistosomula, it has been demonstrated that 42% of the protective effect of immune mechanisms in vaccinated mice occurs in the skin and an additional 17% in the lungs (55). Immunized immune deficient mice have been challenged with cercariae. Athymic nude mice developed no protection whereas nude mice with thymic grafts developed 39.2% protection. This demonstrates that thymic dependent function is necessary for vaccine induced immunity. Destruction of the B cell with anti-μ chain antisera resulted

in no significant protection. Depletion of complement with cobra venom factor did not abrogate vaccine induced immunity (56).

b) Non living vaccines

Attempts at immunization with a non-living preparation of S. Mansoni have been much less successful than irradiated living vaccines. As early as 1959 a soluble extract of S. mansoni adults was reported to give about 50% protection but this has not been confirmed (57). Cercariae antigen given intravenously twenty days prior to infectious challenge offered no protection (58). Homogenates of whole adult worms (55, 59) and freeze thaw extracts of whole adult worms have failed also. Cercariae secreting products and 3M KCl extracts of adult worms give protection in some experiments but this could not be confirmed in repeated experiments (55). Secretory products of adult worms showed no protection (59). Two studies which claim to show protection with non-living preparations use adjuvants which were likely the source of the resistance (59, 60). Protection against Schistosoma mansoni has been recently induced by immunizing mice with sonicated cercariae which were suspended in phenyl methyl sulfonyl fluoride (P.M.S.F.). The mice used were B6D2F1 hybrids which are bred because of their ability to produce IgE. A suspension of the sonicated cercariae was administered with

alum which is an immunological adjuvant. The decrease in adult worm burden was 34 - 90% when compared to adjuvant alone. The protection was paralleled by increases in IgE (61).

Immunization against S. mansoni is partially successful with irradiated larval vaccines but as of yet a reliable reproducible dead vaccine has not been produced. These repeated failures to achieve host protection against S. mansoni infections by immunization with material derived from dead parasites indicate that protective antigens were either not functional or present in amounts too small to be effective (62).

MONOCLONAL ANTIBODIES

The role of antibody in protective immunity against S. mansoni was presented in the section on specific immunity. Until the development of hybridoma technology (63) the target antigens of these antibody dependent interactions could not be identified due to the lack of highly specific antisera. This technique allows investigators to reduce the multispecific response to a complex immunogen, like Schistosoma antigens, to a series of monospecific responses.

Hybrids are obtained by mixing mouse myeloma cells, with normal spleen cells from immunized syngenic mice, in the

presence of the fusing agent polyethelene glycol. The hybrids are grown in media containing hypoxanthine, aminopterin and thymidine. Aminopterin is a folic acid antagonist which prevents de novo synthesis of nucleic acids. Hypoxanthine and thymidine are the substrates for the salvage pathway in nucleic acid syntheses. The myeloma cells used for fusions do not have the enzyme hypoxanthine phosphoribosyl transferase (HPRT) which is necessary for the salvage pathway; they also have been selected because they do not produce immunoglobulin molecules (64). Normal spleen cells have the HPRT enzyme but are unable to survive in tissue culture. The myeloma cells, which are unable to use the hypoxanthine and thymidine in the cell media, would be immortal in non-selective media, but in the presence of aminopterin, die. The only cells which survive are the hybrids of a normal spleen cell which contribute their HPRT and myeloma cells wich contributes its immortality (65). As the myeloma cells used in fusions do not secrete antibodies (64) all antibody production will be that coded for in the genome of the normal spleen cell of the immunized mouse (66). The hybrids producing antibody against the immunizing agent can then be selected and the antibody produced in large quantities in tissue culture or be introduced into the peritoneal cavity of mice and the antibody recovered from the malignant ascites or serum (63, 66).

Monoclonal antibodies have been used to characterize S. mansoni antigens involved in the protective immune response of the host. These antigens are identified by the indirect evidence provided by the passive transfer of immunity with monoclonal antibodies. Three mouse monoclonal antibodies which induce resistance on passive transfer to immunologically naive hosts have been reported (67, 68, 69). One of these monoclonals which was an IgM induced 38% protection, the second an IgG induced 48% protection and the third and IgG_{2b} induced 88% protection. The IgM monoclonal antibody was shown to be positive against fresh schistosomula, adult worms and miracidia by indirect immunofluorescence. In vitro it was also toxic to schistosomula in the presence of complement. Forty-two percent of schistosomula were dead after 98 hours in the presence of the monoclonal antibody and complement while only 3.2% died in the presence of dilute normal serum and complement. The antigens involved were not reported (69). The IgG, monoclonal antibody which was raised against cercariae immunoprecipitated antigens of molecular weight 130,000 and 160,000 (65). The IG_{2b} monoclonal antibody was raised against S. mansoni eggs but also recognized schistosomula surface antigens. A rat IgG_{2a} monoclonal antibody against S. mansoni has also been produced. This monoclonal antibody has been shown to kill 70% of

schistosomula *in vitro*. Control supernatant killed 8%, normal rat serum 6% and immune rat serum 90% of schistosomula. An in vivo experiment showed that intravenous injection of this antibody 4 hours after cercariae challenge gave 50% protection (70). Detergent extracts of schistosomula were immunoprecipitated with this antibody, identifying an antigen of 38,000 molecular weight. Antigens in the range of 30 - 40,000 molecular weight have been precipitated by immune rat, mouse and human serum and may be important. The *in vitro* and *in vivo* studies with this antibody suggest the antigen defined by this monoclonal antibody might correspond to a protective antigen.

AIMS OF PRESENT STUDY

This study was designed to investigate the induction of resistance to Schistosoma mansoni with parasite derived antigens. The specific aims of the study were:

a) To induce protective resistance against S. mansoni in a murine model.

   i) to define a non-living parasite preparation that induces protection in vivo.

   ii) to define and evaluate the protective component.

   iii) to evaluate the mechanism of the protection.

b) To augment the protection produced with parasite preparations by adjuvants.

   i) to define an adjuvant that is effective in induction of resistance subcutaneously.

   ii) to define adjuvants of known chemical structure that induce resistance.

   iii) to combine the parasite preparations within adjuvants to assess the possibility or resistance enhancement.

   iv) to evaluate mechanisms of protection.

c) To define parasite protective antigens using a monoclonal antibody.

   i) raise a monoclonal antibody against S. mansoni.

   ii) characterize the monoclonal antibody and its target antigens.

   iii) evaluate the function of the antibody in vitro and in vivo.

Table 1 (Ref. 22)

Effects of transfer of immune serum with immune spleen cells on Schistosomula recovered from lungs after a challenge infection

GROUP	TREATMENT	LUNG RECOVERY OF SCHISTOSOMULA	p*
Donor (12 week infected)	---	45±7.9	<0.001
Recipient	Normal cells & serum	119±14	---
	Immune serum	67±14	<0.01
	Immune cells	108±14	NS
	Immune cells & serum	79±7.5	<0.01

*Significance of difference from recipients of normal cells and normal serum

Table 2 (Ref. 24)

Cell Mediated Schistosomula Killing
with Complement and Antibody

CELL	HUMORAL FACTOR	% KILLING
Nil	Ab + C	11%
Nil	Media	9%
Neutrophil	Ab	31%
	C	33%
	Ab + C	43%
Eosinophil	Ab	31%
	C	56%
	Ab + C	67%
Monocytes	Ab	22%
	C	31%
	Ab + C	37%

Ab - Antibody

C - Complement

Table 3 (ref. 38)

Protective effect of BCG against challenge with *S. mansoni*

DURATION *S. MANSONI*	SCHISTOSOMULA RECOVERY FROM LUNGS		ADULT WORM RECOVERY	
	Controls	BCG-treated	Controls	BCG-treated
3 days	18±2	6±1		
5 days	166±16	58±6		
7 days	117±10	57±6		
8 weeks			40±4	19±1

Table 4 (Ref. 40)

Protective effect of natural and synthetic mycobacterial products.

SCHISTOSOMULA RECOVERY FROM LUNGS			ADULT WORM RECOVERY
CONTROLS	TREATED	PROTECTION	PROTECTION
185	95*	48%	52%
131	86**	34%	39%

Substance

Natural cord factor *

Trehalose dipalmitate **

Table 5 (Ref. 41)

Growth of *Listeria* at different times during natural *Brucella* infection in mice.

DAY 4		DAY 18		DAY 35	
Normal	Brucella Infection	Normal	Brucella Infection	Normal	Brucella Infection
1.9×10^{4}*	1.6×10^{4}*	1.9×10^{4}*	17*	1.2×10^{4}*	1.2×10^{3}*

*colony forming organisms

Table 6 (Ref. 43)

Arginase levels in resting and activated macrophages.

	BALB/cJ MACROPHAGES		C57 BL/6J MACROPHAGES	
	Untreated	Treated	Untreated	Treated
Arginase Levels	1.1±0.2 U/ml	0.7±0.2 U/ml	0.9±0.1 U/ml	3.8±0.1 U/ml

Figure 1 (ref. 23)

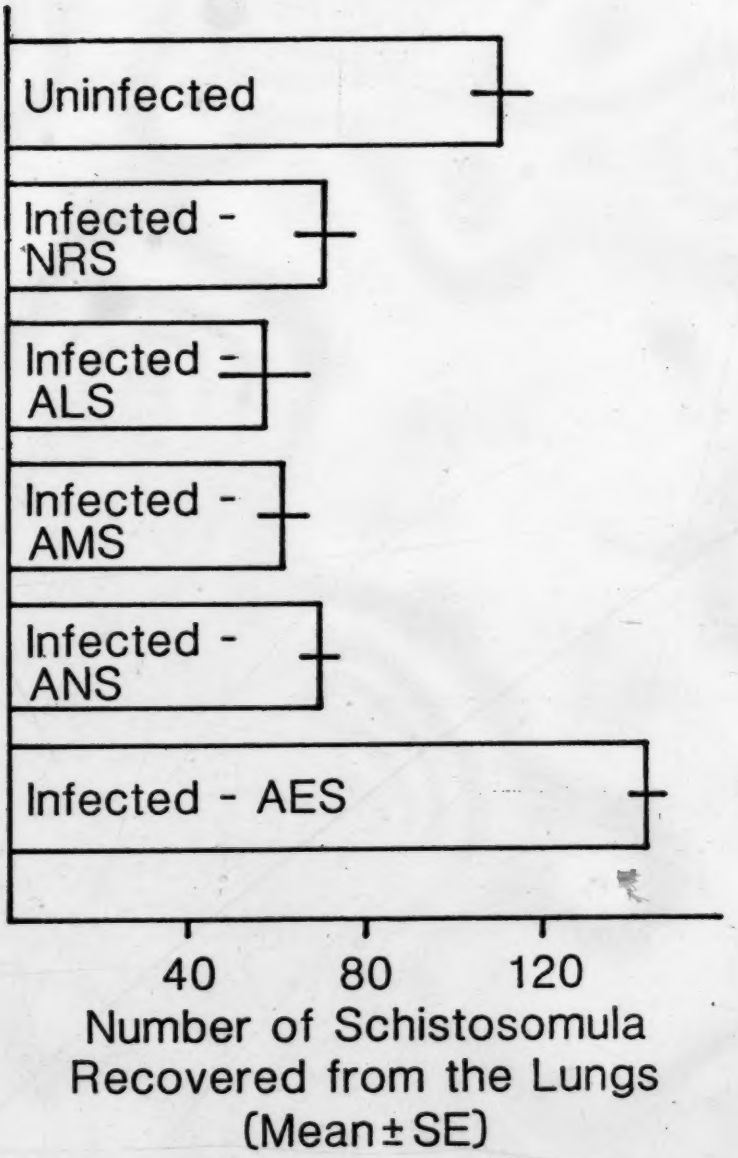


Fig. 1 Recovery of schistosomula 6 days after percutaneous exposure to 500 cercariae of S. mansoni of control, immunologically naive mice, and partially immune mice with chronic schistomiasis treated with NRS, ALS, AMS, ANS, and AES.

NRS - Normal Rat Serum
ALS - Antilymphocyte Serum
AMS - Antimonocyte Serum
ANS - Antineutrophil Serum
AES - Antieosinophil Serum

Figure 2

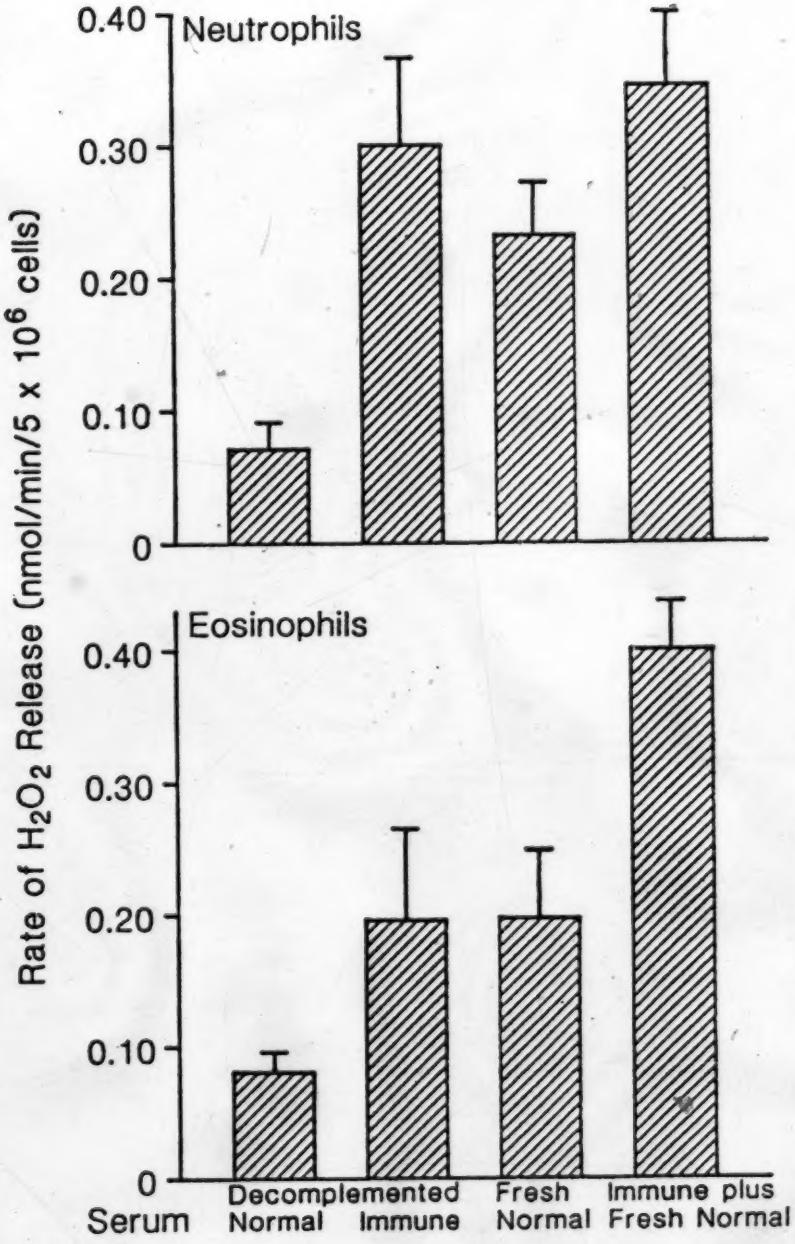


Figure 2 Schistosomula-induced release of H_2O_2 from granulocytes. Five million neutrophils or eosinophils were mixed with 2,500 schistosomula and various serum combinations. After incubation at 37°C for 10 minutes, the rate of H_2O_2 release was measured. Results represent the mean of 8 - 10 experiments.

CHAPTER 2 METHODS

General

a) Laboratory maintenance of S. mansoni life cycle (61). A Puerto Rican strain of S. mansoni is maintained in an albino strain of the snail Australorbis glabratus and an outbred strain of mice, CFI. Mice are injected with 200 cercariae subcutaneously (72). Adult worms were recovered from the portal system of these mice 6 weeks later. To do this the mice were anesthetized with an overdose of sodium pentobarbitol (Abbot laboratories, Chicago) which causes the worm to lose its hold on blood vessel walls. After opening the abdominal cavity a #21 needle was inserted in a hepatic vein which was then perfused and the adult worms washed out through an incision in the portal vein and collected in a beaker containing saline. Eggs were obtained by removing the small intestine from these mice, incubating the intestine for 3 days at 4°C and then grinding them in a blender in the presence of 0.2% trypsin. Through a series of sieving maneuvers intestinal tissue is removed and the eggs isolated. The eggs were hatched in fresh water to yield miracidia. Snails in fresh water were exposed to 10 miracidia overnight at 27°C. Infected snails were then stored in the dark until cercariae were needed. At that time the snails were exposed

to visible light resulting in the shedding of cercariae. Thus adult worms, eggs, miracidia and cercariae were readily available for experimental use.

b) Schistosomula Transformation

Cercariae were transformed to schistosomula by *in vitro* penetration of isolated mouse skin (73). After shaving the abdomen of sacrificed CFI mice the skin was removed and cleared of subcutaneous fat. The skin was then positioned between the chambers of a schistosomula tube (see figure 3). The lower chamber contained a physiological media (Earles Lactalbumin 0.5% for Melnick Monkey Kidney Medium, Flow Laboratories, Meleen, Virginia), the upper chamber was filled with unchlorinated spring water containing freshly shed cercariae. The schistosomula tube was then incubated in the dark for 2 hours at 37°C. The cercariae would penetrate the skin, lose their tail and the resulting schistosomula formed a pellet at the bottom of the lower chamber where they were available for experimental use.

c) Soluble Antigen Preparations

Soluble antigen preparations were made from the adult worms and the eggs. The soluble worm antigen preparation (SWAP) and soluble egg antigen (SEA) were prepared in a similar fashion. The parasite material was placed in a Tenbrock tissue homogenizer and ground for 30 minutes at 4°C.

The resulting suspension was then centrifuged at 40,000 r.p.m. for 2 hours. The supernatants with their soluble antigens were frozen at -70°C until used.

d) Protein Concentration Determination

Throughout this study all protein concentrations were determined by the method of Lowry et al (74). Phenol gives a colour reaction after protein is treated with alkaline copper. The absorbance of the unknown sample usually diluted by a factor of 10 was measured and compared to a standard curve. A new standard curve was established with each protein concentration determination using bovine serum albumin in concentrations ranging from 50 µg to 1 mg/ml. A 1:1 mixture of 1% $CuSo_4$. 5 H_2O and 2% Na tartrate was diluted by a factor of 50 in 2% Na_2CO_3 in .1N NaOH and 2.5 ml of this mixture was added to each 100 µl standard or unknown sample. After incubation at room temperature for 15 minutes .25 ml of Phenol reagent was added. Thirty minutes later the aborbance at 750 nanometers was recorded.

e) Statistical Analysis of Data

Results are expressed as mean ∓ standard error of the mean (s.e. mean). Significant levels for the difference between groups were estimated using Student's paired or unpaired t tests. The difference between groups was judged to be significant when $p < 0.05$.

INDUCTION OF PROTECTION WITH PARASITE PREPARATIONS

a) Animals

Outbred female CFI mice (Charles River Laboratories) 6 weeks of age (18 - 22 g) were used in all studies assessing induction of protection. They were housed in groups no larger than six per cage and maintained on mouse chow (Ralston-Purina, St. Louis, Mo.) and acidified water.

b) Vaccination Protocol

The mice were treated by injection of the different vaccines into the subcutaneous tissue of their backs. A 1 cc tuberculin syringe and a 25 gauge needle were used. Two weeks after vaccination the mice were percutaneously challenged with schistosomiasis. Control animals were injected with either 150 µl of saline or mineral oil.

c) Vaccine Preparations

i) Freeze thaw schistosomula (FTS)

Freshly transformed schistosomula were obtained and counted. The volume of Earles lactalbumin containing 12,000 schistosomula was placed in small test tubes. The schistosomula were centrifuged at 1,000 r.p.m. for 30 seconds and resuspended in normal saline. The schistosomula were again centrifuged and the excess saline removed. This pellet was then placed in a methanol-dry ice bath which instantaneously froze the pellet. The schistosomula were then

thawed in a 20°C water bath. This freezing and thawing was repeated twice more. The volume of the freeze thaw schistosomula was then adjusted so that 2,000 schistosomula were suspended in 150 µl of fluid.

ii) Mechanical Solubilization

Freeze-thaw schistosomula were prepared as described above, placed in a small pyrex tissue homogenizer, and ground by hand for 7 minutes at 4°C. The homonogenate was suspended in normal saline and centrifuged at 10,000 r.p.m. for 10 minutes. The saline solubilized fraction was collected and the volume adjusted to give a final volume of 150 µl per dose.

iii) Triton Extract

Freshly transformed schistosomula were obtained and placed in a microcentrifuge tube. The schistosomula were centrifuged at 1,000 r.p.m. for 30 seconds and the supernatant discarded. A lysis solution of 1 cc of 1% Triton X-100 (Fischer Scientific, Trenton, New Jersey), 20 µl of 10 mM Iodoacetamide (Sigma, St. Louis, Mo.) and 50 µl of 10 mM phenyl methyl sulfonyl fluoride (P.M.S.F.) was prepared. Extraction of the membrane proteins was then performed by adding 250 µl of lysis solution to the pelleted schistosomula. The schistosomula which were kept on ice were vigourously vortexed in this solution every five minutes for the next one half hour. At the end of this time period the

tube was centrifuged and the lysis solution with the solubilized proteins was removed. The final volume was again adjusted with normal saline to result in a 150 µl dose.

d) Cercarial Challenge

Two weeks after vaccination the mice were challenged with schistosomiasis by percutaneous exposure to cercariae. The volume of spring water containing 500 cercariae was determined within one hour of their being shed by infected snails. The 500 cercariae were transferred into disposable petri dishes (35 X 10 mm) containing 4 ml of unchlorinated spring water. The mice were anesthesized by intraperitoneal injection of sodium pentobarbitol (Abbot Laboratories, Chicago) and their abdomens shaved with an electric razor. They were then placed in a prone position over the Petri dishes with their abdomens dependent in the water. After a 30 minute exposure period the animals were removed from contact with the water and returned to their cages (76).

e) Lung Recovery of Schistosomula(17)

Five days after challenge with infection the mice were sacrificed by cervical dislocation. Immediately after death the thoracic cavity was opened to expose the heart and lungs. Five ml of Hanks balanced salt solution (H.B.S.S. - K.C. Biological, Lenexon, Kansas) containing 10 units of heparin/ml was injected into the right ventricle with a syringe fitted

with a 21 gauge needle. This procedure perfused erythrocytes from the lungs and expanded them for easy dissection. The lungs were rinsed with H.B.S.S. and then minced with fine pointed scissors into pieces aproximately 1.5 - 2 mm^3. After suspension of the lung fragments in 10 ml of H.B.S.S. the vials were capped and incubated at 37°C for 3 hours. Following incubation the contents of the vials were filtered through a 36/square inch stainless steel mesh into a 15 ml conical centrifuge tube. The tubes were stored at 4°C overnight and the following morning all but the final 1 ml was discarded. The final ml which contained the lung schistosomula was placed in a Sedgwick counting chamber and the number of schistosomula were determined.

f) Evaluation of Protection

To determine the percentage protection the following formula was used:

$$\frac{(\text{worms recovered from control animals}) - (\text{worms recovered from treated animals})}{\text{worms recovered from control animals}} \times 100$$

g) Mechanisms of Protection

The mechanisms of protection was assessed in mice immunized with freeze-thaw schistosomula. Five days after percutaneous challenge with cercariae, blood was collected and the serum diluted 1:128. Antibody response against SWAP was

measured using an enzyme linked immunosorbent assay (see ELISA for details). Killing by macrophages from FTS immunized mice was assessed in the same manner as BCG treated mice (see mechanisms of BCG resistance to S. mansoni in this chapter).

AUGMENTATION OF PROTECTION WITH ADJUVANTS

a) Vaccination Protocol Cercariae Challenge, Lung Recovery and Assessment of Protection.

The procedures used in these experiments varied little from the protection experiments using parasite material alone. BCG when used with parasite material was mixed in the same syringe. Other adjuvants which required suspension in oil were given by separate injections but into the same site as the parasite material. The percutaneous cercarial challenge occurred two weeks after treatment and the schistosomula were recovered from the lungs on the fifth day.

b) Adjuvants

Bacille Calmette Guerin (B.C.G.) is a living avirulent strain of Mycobacterium bovis isolated by Calmette and Guerin. The mycobacterium became avirulent after 13 years of passage in a medium containing beef bile. All BCG preparations are developed from the original isolate but due to differences in procedure, culture and genetic drift all strains are not the same. The B.C.G. in this study was lyophilized Tice stain which was reconstituted with 1 cc of

sterile water (Biomedical Research, University of Illinois of Chicago). The dose used was 0.5 ml which is 10^7 colony forming units.

Natural Cord Factor 6-6' trehalose dimycolate was purified and extracted from Mycobacterium bovis and obtained from L. Chedid at Institute Pasteur in Paris. Two hundred micrograms of this adjuvant was suspended in 150 µl of mineral oil.

c) Mechanisms of BCG resistance to S. mansoni

In vitro killing of schistosomula by macrophages from mice immunized with FTS and BCG was assessed. Peritoneal exudate cells were obtained by washing the peritoneal cavity of CFI mice which had been immunized 14 days earlier with FTS and BCG and 3 days earlier been injected intraperitoneally with 2 cc of protease peptone (Delco Labs, Detroit). After collection of the peritoneal exudate cells in R.P.M.I. the cells were incubated in plastic petri dishes for 1 hr. at 37°C. The adherent cells were removed with rubber spatulas and then 2 X 10^5 placed with 50 freshly transformed schistosomula. The schistosomula were incubated overnight at 37°C with the macrophages, 50 µl of heat inactivated fetal calf serum and R.P.M.I. Schistosomula viability was visually assessed at 40 power by noting the ability to schistosomula to move and exclude 0.1% toludine blue.

DEFINITION OF PARASITE ANTIGENS IN THE MONOCLONAL ANTIBODY

a) Animals

Female Balb/c mice (18-20 g) were obtained from Jackson laboratories Bar Harbor, Maine. They were maintained on mouse chow (Ralston Purina, St. Louis, Mo.) and acidified water.

b) Immunization Procedure

Balb/c mice were immunized with an intraperitoneal injection of 4000 schistosomula in a 1:1 (volume/volume) emulsion of schistosomula in saline with Freunds complete adjuvant (Difco Laboratories, Detroit). Three weeks later a second injection of 4000 schistosomula in a 1:1 emulsion with Freunds incomplete adjuvant (Difco Laboratories, Detroit) was given subcutaneously. After confirmation by ELISA that the mouse had an antibody response to S. mansoni, 2000 schistosomula were injected into the tail vein.

c) Fusion of Spleen and Myeloma Cells

Four days after the intravenous injection of schistosomula the mice were sacrificed and under sterile conditions their spleens were removed. The spleens were minced to produce a one cell suspension; connective tissue was separated from the lymphocytes by pipetting and erythrocytes were lysed by hypotonic shock. These spleen cells were mixed with NS-1 cells (American Type Culture Collection) in a ratio of 6×10^5 spleen cells to 4×10^5 NS-1 cells. NS-1 cells

are Balb/c myeloma cells which do not produce immunoglobuin and are HPRT negative. The myeloma cells had previously been put into the log phase of growth by suspending them in fresh culture media. The mixed spleen and NS-1 cells were pelleted together and then suspended in 1 ml of a 50% polyethelene glycol (Curtin Matheson Scientific, Elk Grove, Ill.) in cell free culture medium. The cells were gently stirred for one minute and then over the ensuing two minutes the polyethelene glycol was diluted by adding 2 ml of cell free culture medium. At the end of this time period 8 ml of NS-1 culture medium was added, the cells were pelleted and resuspended in cell free culture media without polyethelene glycol. Aliquots of 100 µl were then placed in microtitre wells where the cells were cultured at 37°C in 4% CO_2. Twenty-four hours after fusion the cells were grown in HAT media which resulted in survival of only the products of the fusion of immune spleen cells and NS-1 myeloma cells. Supernatants of the growing cells were collected and successful clones were identified by enzyme linked immunosorbent assay (ELISA).

d) Enzyme Linked Immunosorbent Assay (ELISA)(75)

Five µl of SWAP (50 µg protein/1000 µl of Phosphate Buffered Saline) was adhered to the wells of polyvinyl chloride microtitre plates (Falcon Microtest III, Becton Dickonson and Company, Oxwood, California) by incubation for 48 hours at

4°C. Reagents for washing, dilution of alkaline phosphatase conjugate and the phosphate substrate were obtained from New England Nuclear, Boston, MA. as part of the monoclonal antibody screening kit. Excess SWAP was removed from the wells and then the wells were washed once with 1% Bovine Serum Albumin in 10 mM Tris HCl pH 8 (ELISA Wash). Bovine serum albumin (180 µl of 5% solution) was incubated in each well at 37°C for 1 hour. This step was to block nonspecific protein binding. The bovine serum albumin was removed and the wells were washed with ELISA wash. Fifty µl of ELISA wash was added to each well and then 50 µl of serum diluted 1/64 or undiluted hybridoma supernatant was added. After 2 hours of incubation at 37°C the serum or supernatant was removed and the wells were again washed three times with ELISA wash. Sheep antimouse immunoglobulin conjugated to alkaline phosphatase (New England Nuclear, Boston) was diluted 1/300 in Tris Buffered saline pH 8.5 and 75 µl added to each well. The plate was incubated at 4°C in a humidifier overnight and then the excess conjugate removed. After rinsing the wells three times with ELISA wash, 60 µl of reconstituted 0.05M p-nitrophenyl phosphate (New Endland Nuclear, Boston) was diluted 1/20 in 10 mM 2 amino methyl proponol, 1 mM $MgCl_2$. One hour later absorbance was read on a Microelisa analyzer (Dynatech, Alexandria, Virgina) at 401 nanometers. Infected

mouse serum and normal mouse serum were used as positive and negative controls. All assays were done in either duplicate or triplicate.

e) Concentration of Monoclonal Antibodies

When necessary monoclonal antibody supernatants were concentrated in stirred ultrafiltration cells (Amicon, Lexington, Ma.). Supernatant was added to the cells which had been fitted with a Diaflo^{R} membrane (Amicon, Lexington, Ma.) with pores which would not allow molecules larger that 10,000 M.W. to pass. The cell was attached to a nitrogen gas cylinder which forced water and small molecules through the membrane thus increasing the concentration of the monoclonal antibody. In these studies the monoclonal antibody was concentrated by a factor of ten.

f) Double Immunodiffusion

The monoclonal antibodies class was determined by visualization of precipitin in agar. The monoclonal antibody which had been concentrated 10 times in an Amicon cell was placed in a well centrally located in an agar gel. Surrounding wells contained antibodies to IgA, IgG, IgG_{2a}, IgG_{2b}, IgG_3, and IgM (Cappele, Cochranville, Pa.). Control monoclonal antibody of IgG_{2a} class was used. The gel was incubated at room temperature in a humidifier and the antibody class was identified by visualization of precipitation at 24 hours.

g) Indirect Immuno-fluorescence

Activity of monoclonal antibodies against schistosomula was assessed by indirect immunofluorescence. One hundred schistosomula in 50 µl of Earles lactalbumin were placed in each conical shaped well of a 96 well microtitre plate (Fischer Scientific, Fair Lawn, New Jersey) and kept at 4°C throughout the assay. Either 50 µl of 10 fold concentrated supernatant or serum diluted 1/100 was added and incubated for 45 minutes on a rotary plate shaker (Dynatech microshaker, Alexandria, Virginia). The plate was then centrifuged, the supernatant removed and the schistosomula resuspended in phosphate buffered saline. This washing procedure was repeated twice more to remove antibody not adherent to the schistosomula. Fifty µl of a 1/20 dilution of fluorscein labelled goat antimouse IgM (Capelle Cochranville, Pa.) was added to each well and incubated on the plate shaker for 45 minutes. The schistosomula were then washed three times as previously and then placed on a microscope slide. The fluorescence was noted by direct observation under ultraviolet light. Fluorescence was graded as negative, + for patchy external fluorescence, ++ for fluorescence on the entire limiting membrane and +++ when the body of the schistosomula fluoresces also.

h) Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A discontinuous SDS-PAGE system (77) was used to separate soluble S. mansoni proteins on the basis of molecular weight SWAP was treated with a buffer (0.125 m tris HCL pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and heated in a boiling water bath for 5 minutes. This process yields unfolded peptides with negative charges proportional to their length thus allowing separation on the basis of molecular weight alone.

The stacking gel was 4% acrylamide (0.5 m tris HCl pH 6.8, 4% acrylamide, 2.7% bis acrylamide*). The running gel was 10% acrylamide (1.5 m tris HCl ph 8.8, 10% acrylamide, 2.7% bis acrylamide*). The polymerization of the gels was initiated with 10% ammonium persulphate and 0.2% N N N'N' tetramethylethylmedianne*. The gels and electrodes were submerged in a tank buffer (0.25 m tris pH 8.3, 0.192 m glycine and 0.1% SDS*). SWAP (35 µg) was added to the wells as were high and low molecular weight standards. Electrophoresis was run at a constant current of 30 milliamps for 3.5 to 4 hours. The gel was cut and the portion containing the molecular weight standards was stained with Coumassie blue. After destaining the gel the bands from the molecular weight standards were visible and the distance from the origin of the

separating gel to the center of each band was measured. A standard line was established, the co-ordinates being the log of the molecular weight and the distance from the origin of the separating gel. This allows the determination of the molecular weight of unknown protein bands. The portion of the gel with the parasite proteins was used in the Western blot. (*NOTE. All these reagents were obtained from BioRad - Laboratories, Richmond, California).

i) Western Blot

Blotting is the process of transferring macromolecules from gels to an immobilizing matrix (78). The Western blot is based on the electrolution of proteins from polyacrylamide gels on to nitrocellulose paper(Schleicher & Schuell, Keene, N.H.) which is sandwiched between wet blotting paper, supporting porous pads, and solid plastic grids. The proteins are eluted as anions therefore the nitrocellulose paper is placed on the anode side of the transfer tank, between the gel and the anode. The electrodes are platinum wires strung in a grid 1 cm apart which yields a uniform electric field (see figure 4).The tank buffer is 0.2m Tris, 0.15 M glycine in 20% methanol. The methanol stabilizes the gel and increases the binding of protein to the nitrocellulose paper however it reduces elution efficiency therefore electroelution was carried out for 16 hours at 50 volts.

After the transfer was complete the nitrocellulose paper was cut into strips corresponding to the lanes in the gel that contained SWAP (79). The strips were placed in small plastic trays containing a blocking solution (1% bovine serum albumin, 1% fetal calf serum in PBS tween 0.5%) and incubated for 60 minutes at 37°C on a rocking platform. After washing the paper three times with PBS/0.5% Tween, 10 ml of undiluted monoclonal antibody supernatant directed at S. mansoni was added to one tray and a control monoclonal antibody of the same class but directed against tuberculosis was added in identical conditions. The nitrocellulose paper with the adherent SWAP was incubated with the antibody for 3 hours at room temperature on the rocking platform. After washing 3 times with PBS/0.5% tween, 10 ml of goat antimouse IgM, conjugated to alkaline phosphatase (Capelle Cochranville, Pa.) diluted 1/300 in PBS/0.5% Tween, was added and the blot was incubated at room temperature on the rocking platform. The paper was then washed 3 times with PBS/0.5% tween, twice with PBS/0.5% tween with 0.3% SDS and finally with 0.15 M veronal acetate buffer (0.15 M sodium diethyl barbiturate, Fischer Scientific Fair Lawn, New Jersey - 0.15 M acetic acid pH 9.6). The phosphate reaction solution (20 µl of $MgCl_2$; 1 mg of p-nitro-blue tetrazolium U.S. Biochemical Corporation,

Cleveland, Ohio in 1 ml veronal acetate buffer; .1 ml 5-Bromo -4 Chloro 3- indoyl phosphate - Biochemical Corporation, Cleveland, Ohio in methyl formamide - Sigma, St. Louis, Mo.) was added to the nitrocellulose. The nitrocellulose paper was then incubated at 37° and protein bands identified by the monoclonal antibodies were visualized and the molecular weight determined by comparing to the standard curve described in section h).

j) Monoclonal Antibody Activity

i) in vivo

Monoclonal antibodies were concentrated 10 fold in an ultrafiltration cell. CFI mice were injected intraperitoneally with 1 ml of the concentrated monoclonal antibody supernatant. The following day these mice were challenged with cercariae as described earlier (see cercarial challenge). After recovery from anesthesia 0.5 ml of supernatant was injected intraperitoneally and this was repeated the following day. Five days after cercarial challenge the lung schistosomula were determined by the methods described in section 2e.

ii) In vitro

In vitro killing of schistosomula by peritoneal exudate cells were assessed in the presence of monoclonal antibody, with and without complement. The cells were obtained by

washing the peritoneal cavity of CFI mice 3 days after intraperitoneal injection of 2cc of protease peptone (Delco Labs, Detroit). The peritoneal exudate cells were collected by centrifugation at 1000 r.p.m. for 5 minutes and washed twice in R.P.M.I. (KC Biological - Lenexa, Kansas). The schistosomula were freshly transformed by in vitro skin penetration. Fifty schistosomula were placed in test tubes with 2×10^5 peritoneal exudate cells, monoclonal antibody and either fresh mouse serum, which contains complement, or mouse serum which was heated to 56°C to inactivate complement. The final volume was made up to 0.5 ml by adding R.P..M.I. The schistosomula were incubated overnight and viability was visually assessed at 40 power by noting the ability of schistosomula to move and exclude 0.1% toludine blue.

Figure 3

In vitro transformation of schistosomula.

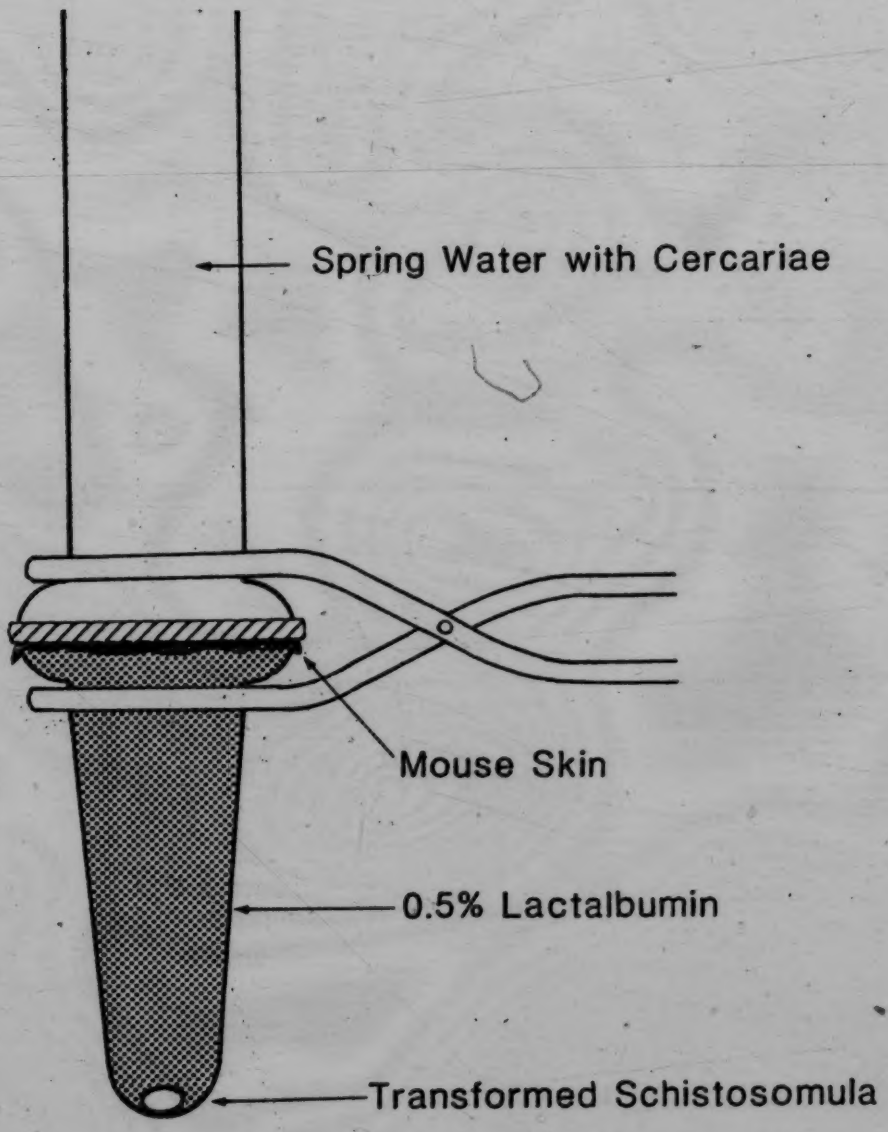


Figure 4

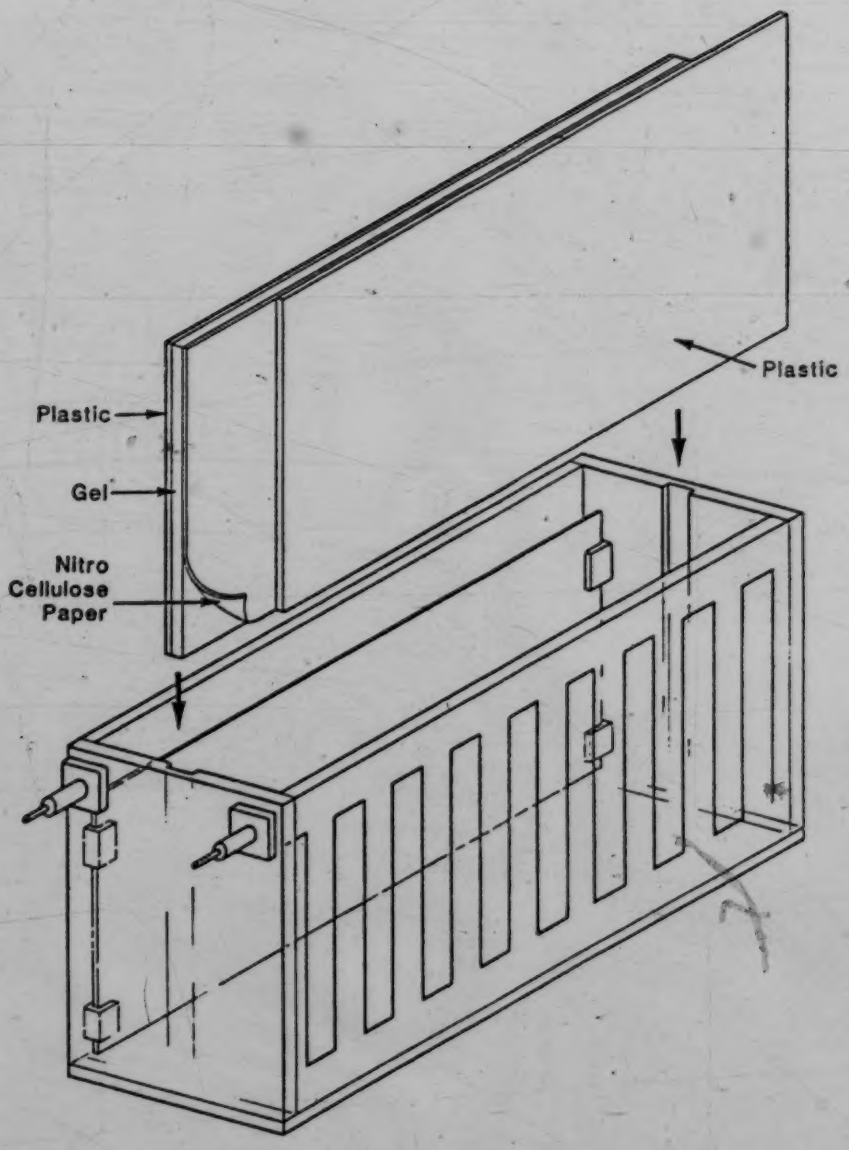


Figure 4 A diagram of the electrophoretic transfer apparatus with one piece of plastic displaced to show the paper and gel.

CHAPTER 3 RESULTS

Induction of Resistance with Parasite Preparations

a) Freeze Thaw Schistosomula

Protection against S. mansoni as measured by lung recovery of schistosomula in six experiments ranged from 16 to 47%. The mean anti-schistosomula protection conferred in the six experiments was 26∓5% (see Table 7). There was a significant difference between the recovery of lung schistosomula from FTS immunized and control mice. The average lung recovery from FTS mice was 102∓4 schistosomula versus 137∓7 larvae for control mice ($P < .001$). Experiments in which repeated doses of FTS were given, or different intervals between immunization and challenge with cercariae were used, did not promote the protection.

b) Mechanical Schistosomula Extract

Protein was extracted into saline by mechanical disruption of freeze thawed schistosomula. The protein extracted from 2000 FTS (35 µg) was injected into groups of mice. No protection was conferred by this preparation as shown in Table 8. Increasing the dose to 75 µg/animal did not result in protective immunity and repeated doses of 35 µg at weekly intervals for 4 weeks also proved ineffective.

c) Triton Schistosomula Extract

Protein was extracted from schistosomula using a solution containing the detergent Triton X-100. The protein from 2000 (40 μg), 5000 (100 μg) and 10,000 (200 μg) schistosomula was injected into groups of mice. As shown in Table 9 significant protection was conferred by the extract from 5000 schistosomula. The lung recovery from these mice was 115∓11 versus the control of 156∓9 ($P < 0.02$). The antischistosomula protection conferred was 25.8%. No protection was conferred by lower or higher doses administered.

d) Mechanisms of Protection

The antibody level in mice immunized with freeze thaw schistosomula was compared to control mice using ELISA. The absorbance level in immunized mice was .323 as compared to 0.024 in control mice. In a second experiment immunized mice had a mean level of .139 while control mice had a level of 0.06. The role of the macrophage in the protection induced by FTS was also evaluated. Peritoneal macrophages from control mice killed 11∓3% of schistosomula. Macrophages from mice treated with FTS killed 15∓3%. The killing of schistosomula from FTS immunized was not statistically different from the control value (see Table 10).

AUGMENTATION OF PROTECTION CONFERRED BY PARASITE MATERIAL WITH ADJUVANTS

a) B.C.G.

Using BCG as an adjuvant, protection was added to the protection conferred by F.T.S. The mean protection of BCG and FTS was 62% while FTS alone gave 19% protection and BCG alone gave 44% protection. The lung recovery of schistosomula from FTS and BCG treated mice was 60∓7 larvae versus 114∓7 for FTS ($P < .001$) As shown in Table 11 the protection conferred by FTS and BCG was significantly greater from BCG alone, FTS alone and control.

b) Chemically Defined Adjuvants

Attempts to augment the protection conferred by F.T.S. with defined adjuvants were unsuccessful. Trehalose dipalmitate (TDM) conferred protection alone but the addition of F.T.S. did not increase this protection. Neither natural cord factor or murabutide increased the protection conferred by F.T.S.

c) Mechanism

The role of the macrophage in BCG induced resistance was evaluated by assaying in vitro killing of schistosomula (see Table 12). Macrophages from control mice killed 11∓3% while macrophages from mice treated with BCG killed 28∓8%. This was a significant increase in killing. Mice immunized with BCG

and FTS killed 32±6% schistosomula. There was no significant increase in killing when FTS was added to BCG in the immunization regimen.

DEFINITION OF PARASITE ANTIGENS WITH A MONOCLONAL ANTIBODY

a) Selection of Mice as a Spleen Cell Source

Enzyme linked immunosorbent assay (ELISA) was performed on serum from Balb/c mice immunized with schistosomula. Two mice were selected for the fusion because their antibody level was greater than that seen in infected mice (see Table 13).

b) Fusion of Spleen and Myeloma Cells (Fusion 31)

57 X 10^6 cells were obtained from one spleen and 39 X 10^6 cells were obtained from the second spleen. These 96 X 10^6 spleen cells were fused to 57.6 X 10^6 NS-1 cells. 147 colonies resulted from this fusion and of these 125 produced enough supernatant to be screened for anti-schistosoma activity.

c) Screening of Fusion 31

The 125 colonies were screened for antibody activity against SWAP using the ELISA. The mean optical density of the duplicate assays was determined and the collected values are shown in a histogram (see Figure 5). The majority of fusions had absorbance levels lower than the .346 noted for normal mouse serum. Seven clones diluted 1:2 had activity equivalent to serum from 18 week infected mice which had been diluted 1:128 (11. Absorbance = 1.4).

d) Characterization of Monoclonal Antibody 31-3 B6

One of the seven clones which produced a high level of antibody was selected for detailed characterization. The antibody class was determined by double immunodiffusion of the supernatant that had been concentrated 10 fold. The antibody was shown to be an IgM. Repeated ELISA against SWAP confirmed the continued activity of 31-3 B6 (see Table 14). This IgM antibody was also assayed against Soluble EGG Antigen (SEA) on an ELISA system. As shown in Table 15 the antibody also demonstrated activity against egg antigen.

e) Indirect Immunofluorescence

31-3 B6 was assayed by indirect immunofluorescence to determine whether or not it had surface binding activity against schistosomula. As shown in Table 16 this antibody had activity against the entire limiting membrane of three hour schistosomula.

f) Antigen Identification

The antigen recognized by the antibody was identified on western blot. The SDS-PAGE molecular weight standards were plotted on a graph (see Figure 6) to establish the relationship between the distance from the origin of the electrophoresis, that a protein band is located and the molecular weight of that protein. On western blot one protein was recognized by 31-3 B6 and no bands were recognized by control

anti-tuberculosis monoclonal antibody (see Figure 7). The band was 9.3 cm from the origin which represents an antigen of apparent molecular weight of 35,000.

g) Function of the Antibody

i) In Vivo

31-3 B6 was given intraperitoneally to immunologically naive mice. Lung recovery of schistosomula was used to compare resistance between treated and control mice. The treated mice showed a recovery of 104∓5 schistosomula while the control value was 148∓7 ($P < .01$). The protection conferred by passive transfer of 31-3 B6 was 30.5% (see Table 17).

ii) In Vitro

Killing of schistosomula with 31-3 B6 was assessed in vitro in a cell free system and in the presence of peritoneal excudate cells. The monoclonal antibody alone did not significantly increase schistosomula killing (see Table 18). In the presence of complement the monoclonal antibody killed 28∓1% of schistosomula in contrast to 16∓2% in media alone. Complement alone does not affect schistosomula death. In vitro killing of schistosomula in the presence of 31-3 B6 and peritoneal exudate cells was assessed (see Table 19). Peritoneal exudate cells effected 25∓4% killing when no opsonin was present. The monoclonal antibody significantly

increased killing to 29±3% and the monoclonal antibody in the presence of complement increased killing to 46±5%. The increase effected by complement and antibody over antibody alone was also statistically significant (see Table 19).

Table 7

Recovery of schistosomula 5 days after percutaneous exposure to 500 cercariae of S. mansoni of control and Freeze Thaw Schistosomula treated mice.

LUNG RECOVERY				
Experiment	No. of Mice in each group	Control	FTS	Protection
1	5	134±9	111±12	16%
2	5	146±18	114±6	22%
3	5	190±19	100±10	47%
4	5	112±4	88±7	21%
5	5	105±12	77±5	29%
6	5	137±4	115±9	17%
Mean of Six Experiments		137±7	102±4	26±5%*

*p <.001

Table 8

Recovery of schistosomula 5 days after percutaneous exposure to 500 cercariae of S. mansoni of control and mechanical schistosomula extract treated mice.

		LUNG RECOVERY			
Experiment	No. of mice in each group	Control	Mechanical Extract	Protection	P value
1	5	125±10	117±16	6%	NS*
2	5	84±6	94±6	< 0	NS

*NS - non significant

Table 9

Recovery of schistosomula 5 days after exposure to 500 cercariae of S. mansoni of control and Triton Schistosomula extract treated mice.

Treatment	No. of mice in each group	Lung Recovery	Protection	P value
Control	5	156±9		
Extract 2,000	5	165±5	< 0	N.S.*
Extract 5,000	5	115±11	25.8%	< .02
Extract 10,000	5	136±10	12%	.2 (NS)

*N.S. - non-significant

Table 10

In vitro killing of schistosomula by peritoneal macrophages from mice immunized with freeze thaw schistosomula.

Treatment	No. of mice in each group	% killing	P value
Control	5	11±3	---
FTS	5	15±3	N.S.*

*N.S. - not significant

Table 11

Recovery of schistosomula 5 days after percutaneous exposure to 500 cercariae of S. mansoni of control, Freeze Thaw Schistosomula treated, BCG treated, and BCG and F.T.S. treated mice.

Treatment	No. of mice	Lung Recovery	% Protection	P value
Control	10	141±11	---	
FTS	10	114±7	19	<.05
BCG	10	77±6	44	<.001
FTS+BCG	10	60±7	62	<.001

Table 12

In vitro killing of schistosomula by peritoneal macrophages from mice treated with BCG and FTS.

TREATMENT	% KILLING	P VALUE
Control	11±3	---
BCG	28±8	<.05
BCG±FTS	32±6	<.05

Table 13

Antibody levels in Immunized Balb/c mice, absorbance measured at 401 nanometers on ELISA with SWAP. (*Mice used in fusion.)

	Absorbance
Normal mouse serum	.261
18 week infected mouse serum	.524
immunized mouse - A_1	.348
immunized mouse - A_4	.319
immunized mouse - B_1	.615*
immunized mouse - B_2	.714*
immunized mouse - B_3	.576

Table 14

Antibody level of 31-3 B6 monoclonal antibody. Absorbance measured at 401 nanometers on ELISA with SWAP.

Antibody Source	Optical Density
Normal Mouse Serum	.346
18 week infected mouse serum	1.402
31-3/B6 monoclonal antibody	1.476

Table 15

Antibody level of 31-3/B6 monoclonal antibody. Absorbance measured at 401 nanometers on ELISA with SWAP and S.E.A.

ANTIBODY SOURCE	OPTICAL DENSITY	
	SEA	SWAP
Normal mouse serum	.073	.021
Immune mouse serum	.692	.326
31-3/B6	.429	.384

Table 16

Determination of surface activity of 31-3/B6 monoclonal antibody by indirect immunofluorescence.

ANTIBODY SOURCE	FLUORESCENCE
Normal mouse serum	---
24 week infected mouse serum	+++
31-3/B6 monoclonal	++

--- negative

\+ patchy immunofluorescence

++ entire limiting membrane fluoresces

+++ limiting membrane and body fluoresces

Table 17

Recovery of schistosomula after percutaneous exposure to 500 cercariae of S. mansoni of control and 31-3/B6 monoclonal antibody treated mice.

TREATMENT	NO. OF MICE	LUNG RECOVERY	% PROTECTION	P VALUE
Control	4	148±7	---	
31-3/B6	3	104±5	30	<.01

Table 18

In vitro killing of schistosomula with media, monoclonal antibody 31-3/B6 and monoclonal antibody with complement.

OPSONIN	% KILLING	P VALUE
media control	16±2	---
31-3/B6	24±3	N.S.
31-3/B6 and complement	28±1	$p<.01$

Table 19

In vitro killing of schistosomula by peritoneal exudate cells, mediated by 31-3/B6 monoclonal antibody and monoclonal antibody with complement.

OPSONIN	% KILLING	P VALUE
nil	25±4	---
31-3/B6	29±3	<.025
31-3/B6 and complement	46±5	<.025

Figure 5

Results of Fusion 31

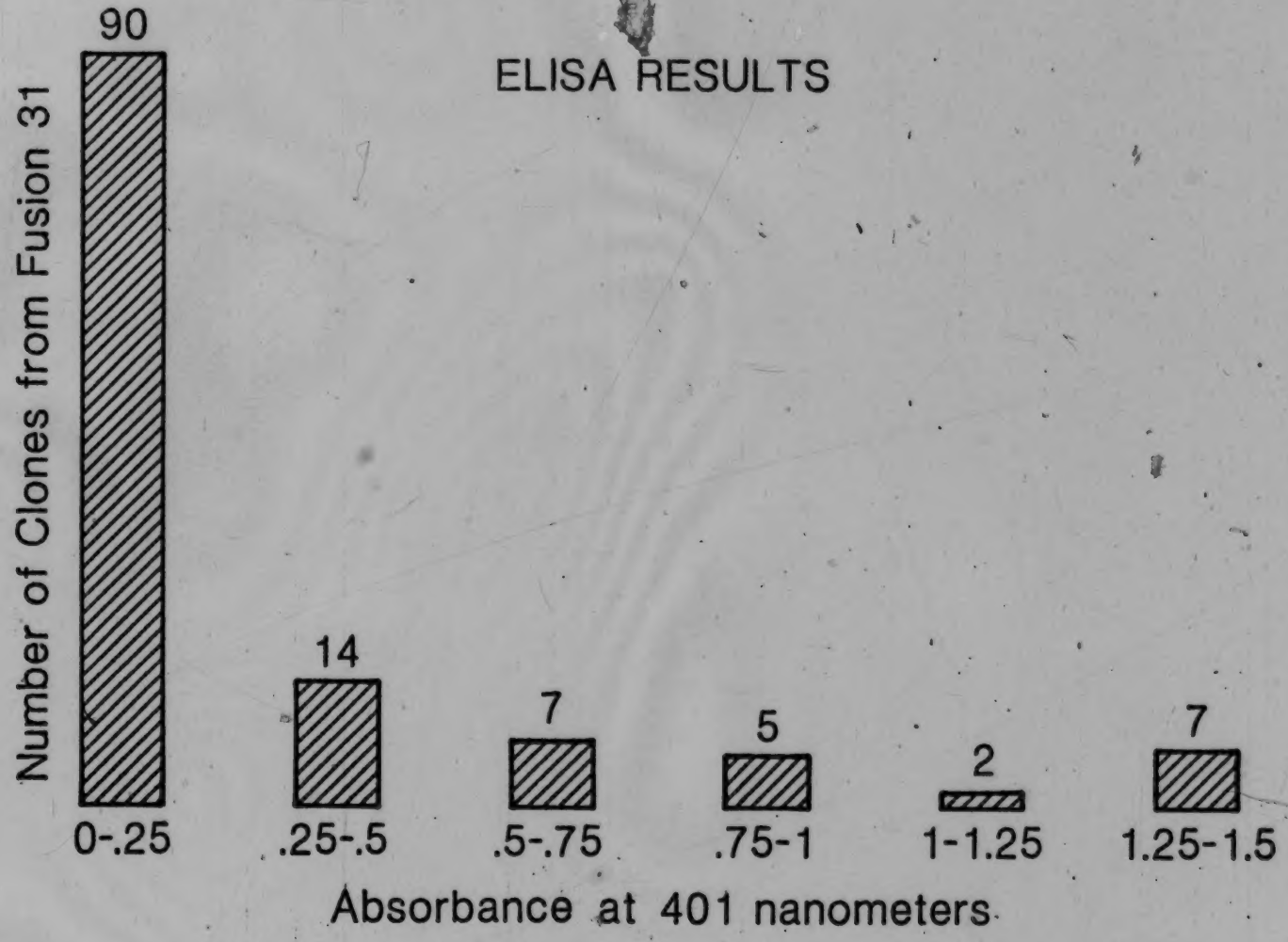


Figure 6

Molecular Weight Linear Regression

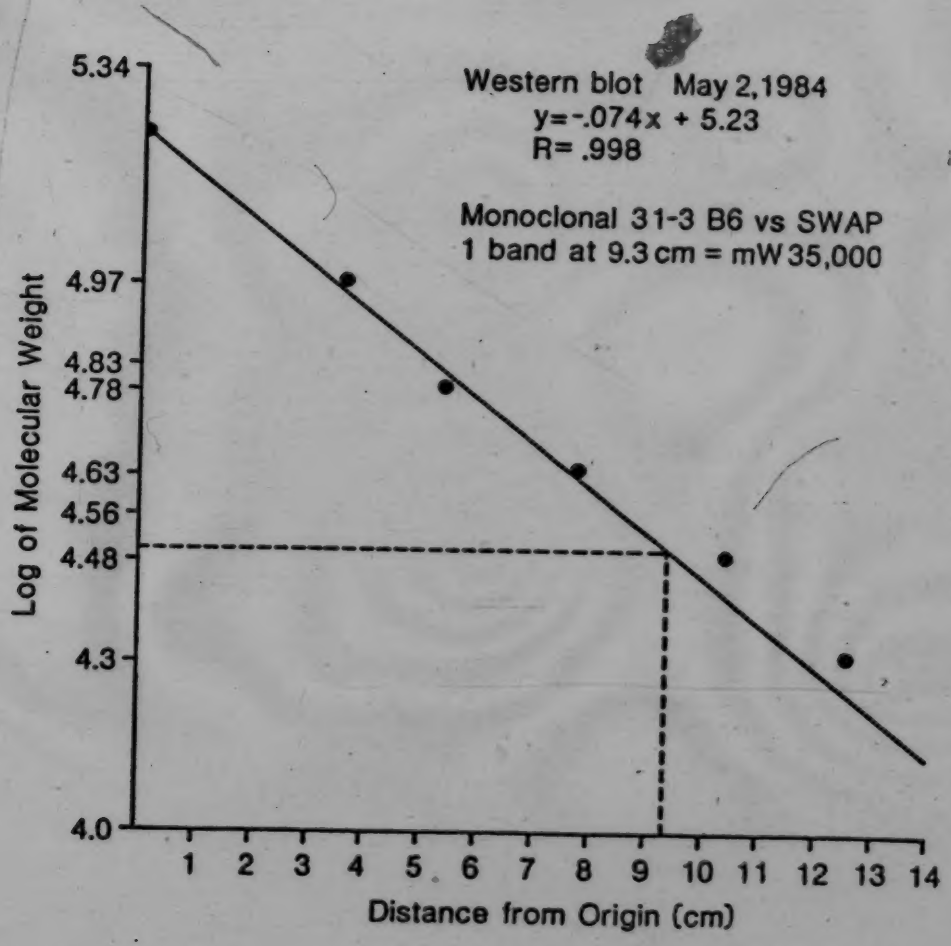
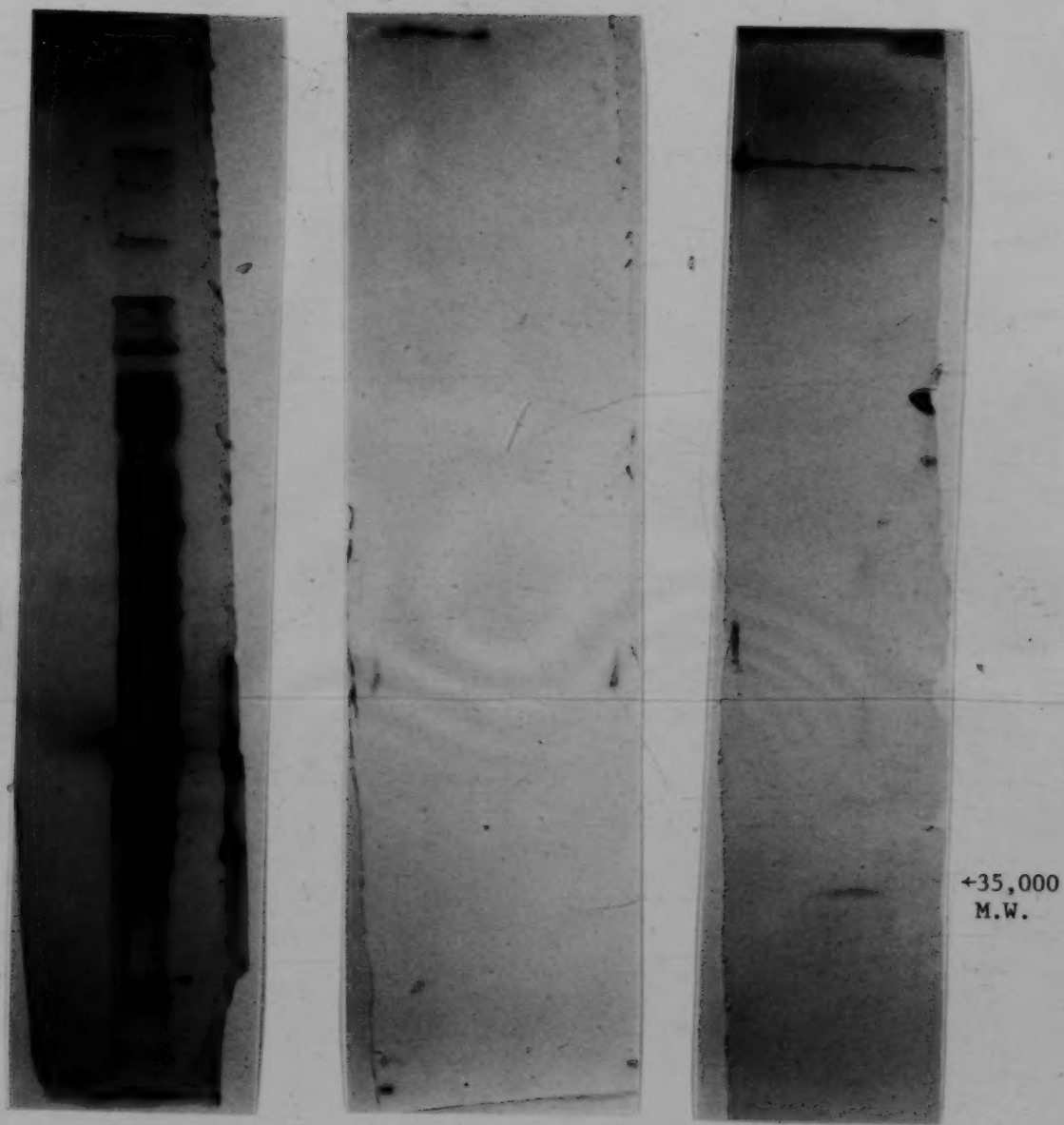


Figure 7

Antigen Defined by 31-3/B6 Monoclonal
Antibody on Western Blot



CHAPTER 4 DISCUSSION

Prevention of infection can be accomplished by avoiding exposure, using chemoprophylactic agents or acquiring resistance to pathogens (80). Avoidance of contact with Schistosoma mansoni in endemic areas is only possible for those individuals whose socio-economic status does not force them to use contaminated water (10). No chemoprophylactic agents and no vaccines are available to prevent S. mansoni (10). Vaccines prepared from inactivated or killed micro-organisms or antigen extracts from these micro-organisms have been effective in preventing many viral and bacterial infections. Small pox has been eradiated (81) and diseases such as diptheria, (82) tetanus (82) and polio (83) are rarities in the developed world. Recently vaccines against pneumococcus (84), menigococcus (85) and hepatitis (86) have been introduced. In contrast to this progress no vaccines against any parasitic disease have been developed for clinical use (87). In recent decades control of parasitic disease was thought to be possible through control of vectors and treatment of affected individuals. Attempts to eradicate malaria by using this approach, has resulted in the development of resistance to DDT in Anopheles mosquitoes, and chloriquine resistance in Plasmodium (88). The use of

molluscacides in attempts to control Schistosoma have failed (10) and despite the introduction of safe chemotherapy schistosomiasis is spreading (5). The failure of vector and disease control to eradicate parasites has resulted in renewed interest in the development of effective vaccines.

Parasites such as Schistosoma and Plasmodium survive in the presence of host antibodies against them. It has been queried how a vaccine could evoke immunity when natural infection cannot. This pessimism is countered by experimental evidence that immunity to S. mansoni can be induced. Chronically infected mice develop up to 75% resistance to reinfection with S. mansoni (17). Serum from chronically infected mice can transfer this protection when injected into immunologically naive mice. In the case of malaria progress has been so substantial that a vaccine feasible for human use is now considered a probability (87). A vaccine against Plasmodium or Schistosoma must improve upon the level of immunity acquired from natural infection (89). This will require isolation of protective antigens and amplification of the host's immune response with immunopotentiators (87).

Monoclonal antibodies have been used to identify protective antigens which might be useful in a vaccine (65, 67, 69, 66)) against S. mansoni. Schistosoma can not multiply in vitro, therefore it is difficult to obtain the large

quantities of parasite material required to purify these antigens by conventional means (90). Large quantities of antigens can now be manufactured in vitro using recombinant DNA technology (87). The gene for a protective antigen on the surface of the merozoite of a simian malaria (Plasmodium knowlesi) has been placed on a plasmid and cloned in E. coli (92). Antigens from S. mansoni have also been produced by in vitro translation of nucleic acids. These proteins have included antigens that are recognized by immune mouse and human serum, but the function of these S. mansoni antigens was not determined (90). These advances in bio-technology which are being used in the development of new viral and bacterial vaccines will be essential for the identification and production of antigens for use in parasite vaccines (87).

Amplification of the host's immune response with immuno-potentiators will be necessary in parasite vaccines (88). This approach is not new; for over 50 years alum has been used as an adjuvant to increase by 10 to 50 fold, the antibody response against diptheria and tetanus toxoids (82). A killed Plasmodium falciparum merozoite antigen administered with a synthetic lipoidal amine (CP 20, 961) as an adjuvant effectively immunized owl monkeys against a lethal P. falciparum infection (91). Monkeys treated with the adjuvant or the antigen alone, died of malaria. The inclusion of an

adjuvant appears to be required to develop effective protective immunity against parasitic diseases. The use of adjuvants in the induction of resistance to S. mansoni has a strong experimental foundation. Non-specific resistance which is the induction of resistance by antigens unrelated to the pathogen has been shown in mice treated with BCG (38) and synthetic products of the mycobacterium cell wall (40). Chronically infected mice treated with mycobacterial adjuvants have shown that the resistance induced non-specifically by the adjuvant can be added to the specific resistance induced by chronic disease (40). The isolation of protective antigens and the amplification of the host's immune response against S. mansoni were the central objectives of this study.

Living cercarial vaccines attenuated by irradiation have induced significant resistance to S. mansoni in laboratory animals (47, 48, 49, 50) and resistance to S. bovis in cattle (66). Despite these successes living vaccines have major disadvantages which make the development of a dead vaccine desirable. A vaccine when developed must be provided to the population at risk (47); survival of a living vaccine produced in the laboratory, until it reaches the field will depend on successful storage and preservation of the parasite (93). Cryopreservation has been effective in maintaining the efficacy of an irradiated schistosomula vaccine. This vaccine

preparation was maintained at -196° for one week and then used to induce protection in mice challenged withh S. mansoni. This protection ranged from 24% to 34% ($P < .001$) (88). The use of these supercooling techniques in areas of the world where even conventional refrigeration is difficult seems quite impractical. Another advantage of a non-living vaccine over the living irradiated vaccines would be that non-living vaccines could be fractionated thus identifying and utilizing protective components while avoiding adverse reactions from other antigens (94, 95).

The data presented in this study demonstrates an effective dead vaccine. Freeze-thaw schistosomula preparation administered to mice induced protection against Schistosoma mansoni as demonstrated by the significant reduction in the number of schistosomula recovered from the lungs of immunized mice 5 days after percutaneous challenge with cercariae. This is an important advance as most previous attempts at immunization with non-living preparations have failed to induce protective immunity (60, 62, 59, 55). The freeze thaw technique was used so as to effect schistosomula killing without drastically changing membrane proteins (96). The choice of schistosomula as the developmental stage to be used in this study was based on the knowledge that it is the first stage of the parasite to interact with the host's immune

mechanisms and because of the successful induction of resistance to S. mansoni with irradiated larval vaccines. It has been shown that larval vaccines do not sensitize the host to form granulomas, suggesting that a vaccine developed from a larval source is less likely to induce this pathologic response (94). The mechanism of the 26% protection induced by FTS was demonstrated to be a specific immune response. Evidence for this was the association of an increased level of antibody in the immunized mice which was not seen in control mice. In vitro killing of schistosomula by macrophages from FTS immunized mice was no different than killing by control macrophages. Previous studies have shown that non-specific resistance to S. mansoni was mediated by activated macrophages. The lack of non-specific killing of schistosomula by macrophages from FTS immunized mice and the increased antibody levels against S. mansoni in FTS treated mice is strong evidence that FTS induces a specific immune response.

After establishing the value of a dead larval vaccine attempts were made to isolate the protective antigen; two approaches were used. The first technique used was the mechanical disruption of freeze thaw schistosomula preparation and the extraction of larval proteins into saline. This technique did extract proteins which were measurable and

visible on polyacrylamide gel electrophoresis but immunization was not effective in inducing protective resistance to S. mansoni. Two possibilities for this failure were considered. One was that the majority of the proteins extracted were matrix proteins rather than membrane proteins. This would imply that the proteins extracted were unlikely targets of the immune response (97). The second possibility was that immunologically relevant proteins were extracted but that they were altered or destroyed during the procedure, perhaps by enzymes released upon disruption of the schistosomula.

The previous factors discussed were taken into account when a second extraction technique was designed. Triton X-100, which is a detergent, was selected to extract proteins because it has been shown, in a study using electronmicroscopy to selectively disrupt the tegument of S. mansoni adult worms while leaving parenchymal tissues intact (98). Phenyl methyl sulfonyl fluoride which binds covalently to functional groups was added to inhibit enzyme degradation (99). Iodoacetamide prevents the formation of new disulphide bonds (100) thus preventing new protein conformations due to the formation of these bonds. 100 µg of Triton Schistosomula Extract induced protection against S. mansoni. This dose induced 25.8% protection as assessed by reduction in recovery of schistosomula from the lungs of mice 5 days after percutaneous

challenge with cercariae. A dose of 40 µg or 200 µg conferred no protection. An antischistosome antibody titre was not demonstrated in the serum of mice treated with Triton Schistosomula Extract. No other investigations into the mechanism of this resistance were performed.

These studies demonstrate that if appropriate precautions are taken to obtain soluble parasite antigens and the dose of antigen is adjusted, the protection induced by the FTS preparations can be duplicated by a cell free parasite derived preparation.

The additive nature of non-specific resistance and specific resistance was exploited by using subcutaneous BCG as an adjuvant with F.T.S. The 26% resistance induced with FTS was increased to 62% when BCG was utilized. The nonspecific mechanism of BCG induced resistance was confirmed by the demonstration of increased killing of schistosomula in vitro by macrophages from mice immunized with B.C.G. This study clearly demonstrates that the additive protection previously described with adjuvants in chronically infected mice can be replicated by using a non-living parasite preparation in combination with an adjuvant. Experiments using three other adjuvants with freeze thaw schistosoma did not induce protective resistance. BCG with Triton schistosomula extract did not show additive resistance in the one experiment in which this was attempted.

A protective schistosomula antigen that may be useful in a vaccine was identified by an IgM monoclonal antibody (31-3/B6). The activity of this antibody against the surface of the schistosomula was confirmed by indirect immunofluorescence and the importance of this antigen to larval viability was demonstrated by the in vitro killing of the larvae by peritoneal exudate cells which was mediated by this antibody. The cell mediated killing of schistosomula by this monoclonal antibody was significantly augmented by complement. The protective value of the antibody was confirmed by the demonstration of a 30% reduction in schistosomula recovered from the lungs of mice which had concentrated monoclonal antibody injected intraperitoneally. The antigen recognized by 31-3/B6 may be of general importance as it was demonstrated to be present in developmental stages of the parasite other than schistosomula. Activity against soluble antigens prepared from S. mansoni adults and eggs was shown on ELISA. The antigen recognized by the monoclonal antibody was shown by western blot to have an apparent molecular weight of 35,000. Two other protective mouse monoclonal antibodies have been reported. One of these, a IgG_{2b} recognizes antigens of 160,000 and 130,000 molecular weight. The other antibody an IgG_1 recognized different antigens of the same molecular weight (68). The antigens recognized by

these other protective mouse monoclonal antibodies are of much higher molecular weight from that recognized by 31-3/B6. A rat monoclonal antibody which recognizes an antigen of apparent molecular weight of 38,000 is also protective against S. mansoni (70). The antigens recognized by protective monoclonal antibodies will be prime candidates for use in vaccines.

The prevention of schistosomiasis mansoni by a practical and safe immunization technique is a desirable goal. Further progress in the development of a non-living vaccine preparation against S. mansoni will simplify the logistics of vaccine delivery to the population at risk. Solubilization of antigens and identification of specific protective antigens should eventually result in a vaccine of high efficacy with few if any side effects. Adjuvants which have been demonstrated to be of value in combination with a non-living vaccine will be just as important as the antigen preparation (87). Chemically defined adjuvants which stimulate both the specific and non-specific resistance would be necessary for an effective Schistosoma mansoni vaccine.

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